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ORIGINAL PAPER

Antiamnesic and Antioxidants Effects of *Ferulago angulata* Essential Oil Against Scopolamine-Induced Memory Impairment in Laboratory Rats

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Abstract *Ferulago angulata* (Apiaceae) is a shrub indigenous to western Iran, Turkey and Iraq. In traditional medicine, *F. angulata* is recommended for treating digestive pains, hemorrhoids, snake bite, ulcers and as sedative. In the present study, the effects of inhaled *F. angulata* essential oil (1 and 3 %, daily, for 21 days) on spatial memory performance were assessed in scopolamine-treated rats. Scopolamine-induced memory impairments were observed, as measured by the Y-maze and radial arm-maze tasks. Decreased activities of superoxide dismutase, glutathione peroxidase and catalase along with increase of acetylcholinesterase activity and decrease of total content of reduced glutathione were observed in the rat hippocampal homogenates of scopolamine-treated animals as compared with control. Production of protein carbonyl and malondialdehyde significantly increased in the rat hippocampal homogenates of scopolamine-treated animals as compared with control, as a consequence of impaired antioxidant enzymes activities. Additionally, in scopolamine-treated rats exposure to *F. angulata* essential oil significantly improved memory formation and decreased oxidative stress, suggesting memory-enhancing and antioxidant effects. Therefore, our results suggest that multiple exposures to *F. angulata* essential oil ameliorate scopolamine-induced spatial memory impairment by attenuation of the oxidative stress in the rat hippocampus.

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Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder, characterized by the pathological accumulation of beta-amyloid peptides, neurofibrillary tangles, synaptic loss, neuroinflammation and oxidative stress, eventually leading to cognitive decline [1].

Acetylcholinesterase (AChE) enzyme catalyzes acetylcholine (ACh) hydrolyzes causing a decrease in ACh amount in the brain. According to cholinergic hypothesis, inhibition of AChE improves cholinergic functions in AD patients. Cholinesterase inhibition is one of the keystone in the treatment of AD and is a promising strategy for the therapy of dementia [2].

A muscarinic receptor antagonist, scopolamine, is provided based on cholinergic hypothesis [3]. Scopolamine has been used as a reference drug for inducing age and dementia related cognitive deficits in healthy humans and animals [4]. It is a well-established amnesic drug [5], impairing learning and memory in rodents and humans, especially in the processes of learning acquisition and short-term memory [6].

It is known that oxidative stress plays an important role in the ethiopathogenesis of AD. There is accumulating evidence suggesting that oxidative stress is an early event in the development of the disease and such oxidative changes are pervasive throughout the body [7]. It has been also proposed that oxidative stress has an important role in modulating signaling pathways leading to cell death [8, 9]. Several studies have reported the presence of elevated DNA, RNA, protein and lipid oxidation in brains of

patients with AD and mild cognitive impairment (MCI) [10–12].

Some *Ferulago* species have been used in folk medicine for their sedative, tonic, digestive, anti-parasitic and aphrodisiac properties [13]. *F. angulata* has already shown to have high antioxidant properties [14]. It has been reported that the hydroalcoholic extract of *F. angulata* can reduce serum levels of total cholesterol, triglycerides, and low-density lipoproteins (LDL) as well as inhibiting lipid peroxidation in Wistar rats [15]. However, there is no study, clarifying the possible cognitive-enhancing and antioxidant potentials of *F. angulata* essential oil in the animal models of AD. Herein, we examined the effects of *F. angulata* essential oil on memory processes as well as the importance of the essential oil in oxidative stress status in the hippocampus of scopolamine-treated rats. Correlation between the behavioral scores and the levels of the main oxidative stress markers from the hippocampus of scopolamine-treated rats, as a result of inhalation of essential oil was also investigated.

Materials and Methods

Plant Materials and Volatile Oil Preparation

Aerial parts of *F. angulata* were collected in the flowering stage near Elazig, Eastern Anatolia, Turkey, in June 2013. The samples of the plants were identified by Prof. dr. Eyup Bagci and a voucher specimen was registered and deposited in the Herbarium of Department of Biology, Firat University for ready reference. The oil was extracted by hydro-distillation for 3 h using a Clevenger-type apparatus. The total essential oil yield was 0.7 % (v/w).

Oil Chromatographic Analyses

Essential oil was analyzed by GC–MS (Agilent 5973 N) and GC-FID (Agilent 6890 GC) with a column of HP-5 MS (30 m × 0.25 mm i.d., film thickness, 0.25 μm) and detector in Plant Products and Biotechnology Research Laboratory (BUBAL), Firat University. The sample was injected by splitting and the split ratio was 1:100. The GC oven temperature was kept at 70 °C for 2 min and programmed to 150 °C at a rate of 10 °C/min and then kept constant at 150 °C for 15 min to 240 °C at a rate of 5 °C/min. Helium was used as the carrier gas at a flow rate of 1 ml/min. MS were taken at 70 eV and a mass range of 35–425. The identification of the compounds was based on comparison of their retention indices (RI), their retention times (RT) and mass spectra with those obtained from authentic Wiley libraries (available through Hewlett Packard) and the literature [16].

Animals

24 Male Wistar rats (3 month old) weighing 250 ± 5 g at the start of the experiment were used. The animals were housed in a temperature and light-controlled room (22 °C, a 12-h cycle starting at 08:00 h) and were fed and allowed to drink water ad libitum. The rats were divided into four groups (6 animals per group): (1) Control group received 0.9 % saline with 1 % Tween 80 treatment; (2) Scopolamine (Sco)—alone-treated group received 0.9 % saline with 1 % Tween 80 treatment, as negative control; (3) Scopolamine-treated group received *F. angulata* essential oil 1 % (Sco + FEO1%) and (4) Scopolamine-treated group received *F. angulata* essential oil 3 % (Sco + FEO3%). Control and scopolamine alone-treated groups were caged in the same conditions but in the absence of the tested essential oil. Rats were treated in accordance with the guidelines of the animal bioethics of the Act on Animal Experimentation and Animal Health and Welfare from Romania and all procedures were in compliance with Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes.

Inhalation Apparatus and Drug Administration

The inhalation apparatus consisted of a Plexiglas chamber (50 cm × 40 cm × 28 cm). Two chambers were used, one for the control and scopolamine alone-treated animals, which were exposed to 0.9 % saline with 1 % Tween 80 solution, and the other one for the experimental animals, which were exposed to *F. angulata* essential oil (1 and 3 %). *F. angulata* essential oil was diluted with 1 % Tween 80 (v/v). *F. angulata* essential oil exposure (200 μl, either 1 or 3 %) was via an electronic vaporizer (Oregon Scientific WS113) placed at the bottom of the chamber, but out of reach of the animals. Regarding concentrations to be used in the pharmacological tests, we selected 1 % essential oil normally used in aromatherapy and a higher concentration (3 %) in order to emphasize the effects [17]. Rats in the *F. angulata* essential oil groups were exposed to oil vapors for controlled 60 min period, daily, for 21 continuous days. 60 min is a suitable inhalation period for the expected effects [18]. Chambers were always cleaned up (10 % ethanol solution). Scopolamine hydrobromide (Sigma-Aldrich, Germany) was dissolved in an isotonic solution (0.9 % NaCl) and 0.7 mg/kg scopolamine was injected intraperitoneally (i.p.), 30 min before the behavioral testing in the Y-maze and radial arm-maze tasks.

Y-maze Task

Short-term memory was assessed by spontaneous alternation behavior in the Y-maze task. The Y-maze used in the present study consisted of three arms (35 cm long, 25 cm high and 10 cm wide) and an equilateral triangular central area. 60 min after the inhalation of *F. angulata* essential oil (FEO1% and FEO3%), rats were placed at the end of one arm and allowed to move freely through the maze for 8 min. An arm entry was counted when the hind paws of the rat were completely within the arm. Spontaneous alternation behavior was defined as entry into all three arms on consecutive choices. The percentage of triads in which the rats entered all three arms, i.e., ABC, CAB, or BCA but not ABB, was recorded as an alternation to estimate short-term memory. The spontaneous alternation (%) for each rat was defined as the ratio of the actual number of alternations to the possible number (defined as the total number of arm entries minus two) multiplied by 100 as shown by the following equation: Spontaneous alternations (%) = [(Number of alternations)/(Total arm entries−2)] × 100 [19, 20]. Spontaneous alternation behavior is considered to reflect spatial working memory, which is a form of short-term memory. The number of arm entries per trial was used as an indicator of locomotor activity. The maze was cleaned with a 10 % ethanol solution and dried with a cloth before the next animal was tested.

Radial Arm-maze Task

The radial arm-maze used in the present study consisted of eight arms, numbered from 1 to 8 (48 cm × 12 cm), extending radially from a central area (32 cm in diameter). The apparatus was placed 50 cm above the floor, and surrounded by various extra-maze visual cues placed at the same position during the study. At the end of each arm there was a food cup that had a single 50 mg food pellet. Prior to the performance of the maze task, the animals were kept on a restricted diet and body weight was maintained at 85 % of their free-feeding weight over a week period, with water being available ad libitum. Before the actual training began, three or four rats were simultaneously placed in the radial arm-maze and allowed to explore for 5 min and take the food freely. The food was initially available throughout the maze, but was gradually restricted to the food cup. The animals were trained for 4 days to run to the end of the arms and consume the bait. To evaluate the basal activity of rats in a radial arm-maze, the rats were given 5 consecutive training trials per day to run to the end of the arms and consume the bait. The training trial continued until all 5 baits have been consumed or until the 5 min have elapsed which have been set as the performance criteria. After adaptation, all rats were trained with 1 trial per day. Briefly, 60 min after the inhalation of *F. angulata* essential oil (FEO1% and FEO3%), each animal was placed individually in the center of the maze and subjected to working and reference memory tasks, in which same 5 arms (nos. 1, 2, 4, 5 and 7), were baited for each daily training trial. The other 3 arms (nos. 3, 6 and 8) were never baited. The selection of the baited arms is based on the fact that animals prefer to solve the maze using an adjacent arm selection strategy. In this case, we altered adjacent arm patterning behavior by only baiting 5 arms (nos. 1, 2, 4, 5, and 7) subjecting animals to change their strategy and avoid the unbaited arms. An arm entry was counted when all four limbs of the rat were within an arm. Measures were made of the number of working memory errors (entering an arm containing food, but previously entered) and reference memory errors (entering an arm that was not baited) [19, 20]. Reference memory is regarded as a long-term memory for information that remains constant over repeated trials (memory for the positions of baited arms), whereas working memory is considered a short-term memory in which the information to be remembered changes in every trial (memory for the positions of arms that had already been visited in each trial). The maze was cleaned with a 10 % ethanol solution and dried with a cloth before the next animal was tested.

Oxidative Stress Assay

After the behavioral tests, all rats were deeply anesthetized (using sodium pentobarbital, 100 mg/kg b.w., i.p., Sigma-Aldrich, Germany), decapitated and whole brains were removed. The hippocampi were carefully excised. Each of the hippocampal samples were weighted and homogenized (1:10) with Potter Homogenizer coupled with Cole-Parmer Servodyne Mixer in ice-cold 0.1 M potassium phosphate buffer (pH 7.4), 1.15 % KCl. The homogenate was centrifuged (15 min at 960×*g*) and the supernatant was used for assays of SOD, CAT, GPX and AChE specific activities, the total content of reduced GSH, protein carbonyl and MDA levels.

Determination of Hippocampal AChE Activity

Activity of acetylcholinesterase (AChE) in the rat hippocampus was determined according to the method of Ellman et al. [21] using acetylthiocholine (ATC) as artificial substrate [22]. The reaction mixture (600 µl final volume) contained 0.26 M phosphate buffer with pH 7.4, 1 mM 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB) and 5 mM ATC chloride. The assay was started by adding supernatant and following the development of the yellow color at 412 nm for 10 min at room temperature. Suitable

controls were performed for the non-enzymatic hydrolysis of ATC. The enzyme activity is expressed as nmol of ACT/min per/mg of protein.

Determination of Hippocampal SOD Activity

The activity of superoxide dismutase (SOD, EC 1.15.1.1) was assayed by monitoring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT). Each 1.5 ml reaction mixture contained 100 mM TRIS/HCl (pH 7.8), 75 mM NBT, 2 μM riboflavin, 6 mM EDTA and 200 μl of supernatant. Monitoring the increase in absorbance at 560 nm followed the production of blue formazan. One unit of SOD is defined as the quantity required to inhibit the rate of NBT reduction by 50 % as previously described by Winterbourn et al. [23]. The enzyme activity is expressed as units/mg protein.

Determination of Hippocampal CAT Activity

Catalase (CAT, EC 1.11.1.6) activity was assayed following the method of Sinha [24]. The reaction mixture consisted of 150 μl phosphate buffer (0.01 M, pH 7.0), 100 μl supernatant. Reaction was started by adding 250 μl H_2O_2 0.16 M, incubated at 37 °C for 1 min and the reaction was stopped by addition of 1 ml of dichromate:acetic acid reagent. The tubes were immediately kept in a boiling water bath for 15 min and the green colour developed during the reaction was read at 570 nm on a spectrophotometer. Control tubes, devoid of enzyme, were also processed in parallel. The enzyme activity is expressed as μmol of H_2O_2 consumed/min/mg protein.

Determination of Hippocampal GPX Activity

Glutathione peroxidase (GPX, E.C. 1.11.1.9) activity was analyzed by a spectrophotometric assay. A reaction mixture consisting of 1 ml of 0.4 M phosphate buffer (pH 7.0) containing 0.4 mM EDTA, 1 ml of 5 mM NaN_3, 1 ml of 4 mM glutathione (GSH), and 200 μl of supernatant was pre-incubated at 37 °C for 5 min. Then 1 ml of 4 mM H_2O_2 was added and incubated at 37 °C for further 5 min. The excess amount of GSH was quantified by the DTNB method as previously described by Sharma and Gupta [25]. One unit of GPX is defined as the amount of enzyme required to oxidize 1 nmol GSH/min. The enzyme activity is expressed as units/mg protein.

Total Hippocampal Content of Reduced GSH

Glutathione (GSH) was measured following the method of Fukuzawa and Tokumura [26]. 200 μl of supernatant was added to 1.1 ml of 0.25 M sodium phosphate buffer (pH 7.4) followed by the addition of 130 μl DTNB 0.04 %. Finally, the mixture was brought to a final volume of 1.5 ml with distilled water and absorbance was read in a spectrophotometer at 412 nm and results were expressed as μg GSH/μg protein.

Determination of Hippocampal Protein Carbonyl Level

The extent of protein oxidation in the hippocampus was assessed by measuring the content of protein carbonyl groups, using 2,4-dinitrophenylhydrazine (DNPH) derivatization as described by Oliver et al. [27] and following the indications of Luo and Wehr [28]. Basically, the supernatant fraction was divided into two equal aliquots containing approximately 2 mg of protein each. Both aliquots were precipitated with 10 % trichloroacetic acid (TCA, w/v, final concentration). One sample was treated with 2 N HCl, and the other sample was treated with an equal volume of 0.2 % (w/v) DNPH in 2 N HCl. Both samples were incubated at 25 °C and stirred at 5 min intervals. The samples were then reprecipitated with 10 % TCA (final concentration) and subsequently extracted with ethanol-ethyl acetate (1:1, v/v) and then reprecipitated at 10 % TCA. The pellets were carefully drained and dissolved in 8 M urea with 20 mM sodium phosphate buffer, pH 6.5. Insoluble debris was removed by centrifugation at 13,000×*g* at 4 °C. The absorbance at 370 nm of the DNPH-treated sample versus the HCl control was recorded, and the results are expressed as nmols of DNPH incorporated/mg of protein based on an average absorptivity of 21 mM^{-1} cm^{-1} for most aliphatic hydrazones.

Determination of Hippocampal MDA Level

Malondialdehyde (MDA), which is an indicator of lipid peroxidation, was spectrophotometrically measured by using the thiobarbituric acid assay as previously described by Ohkawa et al. [29]. 200 μl of supernatant was added and briefly mixed with 1 ml of 50 % trichloroacetic acid in 0.1 M HCl and 1 ml of 26 mM thiobarbituric acid. After vortex mixing, samples were maintained at 95 °C for 20 min. Afterwards, samples were centrifuged at 960×*g* for 10 min and supernatants were read at 532 nm. A calibration curve was constructed using MDA as standard and the results were expressed as nmol/mg protein.

Estimation of Protein Concentration

Estimation of protein was done using a BCA protein assay kit (Sigma-Aldrich, Germany). The BCA protein assay is a detergent-compatible formulation based on bicinchoninic acid (BCA) for the colorimetric detection and

quantification of total protein, as previously described by Smith et al. [30].

Statistical Analysis

The animal's behavioral activities in the Y-maze and the radial arm-maze tasks and the results of biochemical parameter assays were statistically analyzed by one-way analysis of variance (ANOVA) using GraphPad Prism version 6.00 for Windows, GraphPad Software, La Jolla California USA. In order to evaluate differences between groups in the radial arm-maze task, separate repeated-measures ANOVA was calculated on the number of working memory errors and the number of reference memory errors with group (Control, Sco, Sco + FEO1% and Sco + FEO3%) as between-subject factor and days (1–7) as within-subjects factors. All results are expressed as mean ± S.E.M. F values for which $p < 0.05$ were regarded as statistically significant. Significant differences were determined by Tukey's post hoc test. Pearson's correlation coefficient and regression analysis were used in order to evaluate the connection between behavioral measures, the antioxidant defence and lipid peroxidation.

Results

Chemical Composition of the *F. angulata* Essential Oil

The chemical composition of the *F. angulata* essential oil was analyzed by GC–MS/GC-FID. A total of 48 different compounds were isolated which constituted 96.5 % (w/w) of the total essential oil. The principal components of the essential oil were monoterpene hydrocarbons ($C_{10}H_{16}$), including α-pinene (24.10 %), β-pinene (22.70 %), α-phellandrene (12.10 %) and β-phellandrene (20.50 %), which accounted for 79.40 % of the total essential oil.

Effect of the *F. angulata* Essential Oil on Spatial Memory in Y-maze Task

The one-way ANOVA revealed significant overall differences between all groups $F(3,36) = 2.77$, $p < 0.01$) on spatial working memory, as evidenced by the spontaneous alternations percentage (Fig. 1a). The Tukey's post hoc analysis revealed significant differences between control versus Sco groups ($p < 0.001$) and Sco and Sco + FEO3% groups ($p < 0.0001$) for the spontaneous alternations percentage (Fig. 1a). Non-significant differences between the scopolamine treated-groups exposed to FEO1% and FEO3% on the spontaneous alternation percentage were observed.

The changes in the spontaneous alternation percentage of both Sco + FEO1% and Sco + FEO3% groups are related to the changes in motor activity, as evidenced in the Y-maze task by the number of arm entries ($F(3,36) = 8.10$, $p < 0.001$) (Fig. 1b).

Effect of the *F. angulata* Essential Oil on Spatial Memory in Radial Arm-maze Task

To investigate whether the inhalation of the *F. angulata* essential oil affects the spatial memory formation, the rats were further evaluated in the radial arm-maze task.

The one-way ANOVA revealed significant overall differences between all groups ($F(3,36) = 5.50$, $p < 0.001$) on working memory, as evidenced by the number of working memory errors (Fig. 2a). The Tukey's post hoc analysis revealed significant differences between control versus Sco groups ($p < 0.001$), control versus Sco + FEO1% groups ($p < 0.0001$), control versus Sco + FEO1% groups ($p < 0.01$), Sco and Sco + FEO3% groups ($p < 0.0001$) for working memory errors (Fig. 2a). Additionally, repeated-measures ANOVA revealed a significant time difference ($F(6,140) = 2.56$, $p < 0.01$) and a significant group difference ($F(3,140) = 10.65$, $p < 0.0001$) for working memory errors (Fig. 2a).

The one-way ANOVA revealed significant overall differences between all groups ($F(3,36) = 4.43$, $p < 0.0001$) on reference memory, as evidenced by the number of reference memory errors (Fig. 2b). Tukey's post hoc analysis revealed non-significant differences between groups for reference memory errors (Fig. 2b). Additionally, repeated-measures ANOVA revealed a significant time difference ($F(6,140) = 4.39$, $p < 0.0001$) for reference memory errors (Fig. 2b).

Effect of the *F. angulata* Essential Oil on AChE Activity

For the AChE specific activity estimated in the rat hippocampal homogenates, one-way ANOVA revealed a significant overall differences between groups ($F(3,36) = 41.06$, $p < 0.0001$) (Fig. 3a). Additionally, Tukey's post hoc analysis revealed significant differences between control and Sco + FEO1% groups ($p < 0.0001$), control and Sco + FEO3% groups ($p < 0.0001$), Sco and Sco + FEO1% groups ($p < 0.001$) and Sco and Sco + FEO3% groups ($p < 0.0001$) for AChE specific activity (Fig. 3a).

Effect of the *F. angulata* Essential Oil on SOD, GPX and CAT Activities

For the SOD specific activity estimated in the rat hippocampal homogenates, one-way ANOVA revealed a

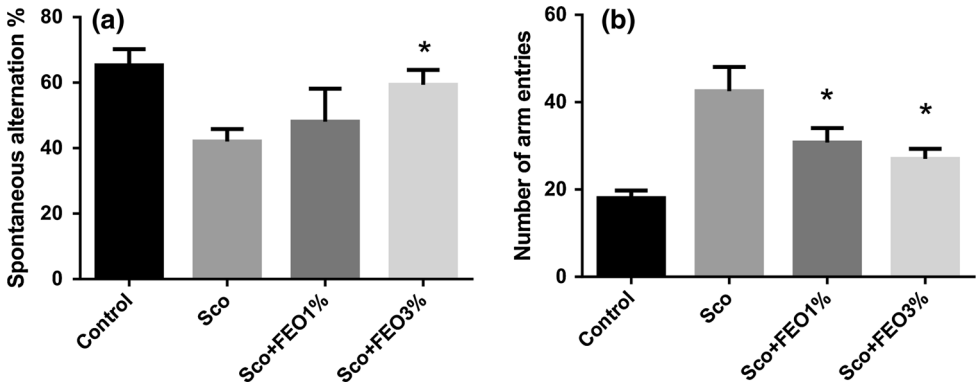


Fig. 1 Effects of inhaled *Ferulago angulata* essential oil (FEO1% and FEO3%) in the Y-maze on spontaneous alternation % (**a**) and the number of arm entries (**b**) in the scopolamine (Sco)-treated rats. Values are mean ± S.E.M. (n = 6 animals per group), *$p < 0.0001$ versus scopolamine alone-treated group

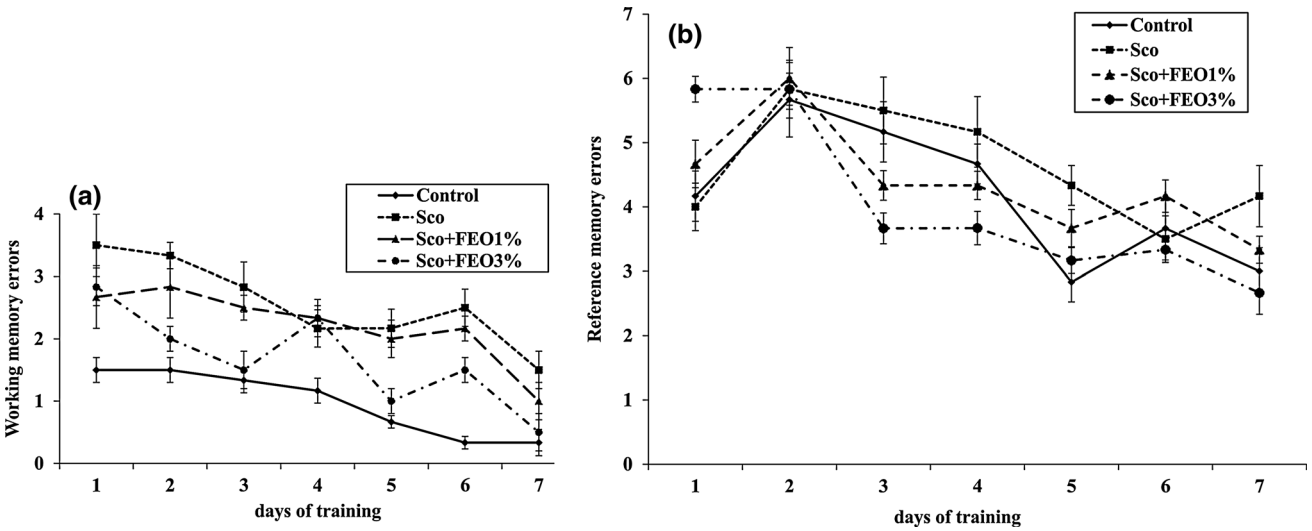


Fig. 2 Effects of inhaled *Ferulago angulata* essential oil (FEO1% and FEO3%) on the working memory errors (**a**) and the reference memory errors (**b**) during 7 days training in the radial arm-maze in the scopolamine (Sco)-treated rats. Values are mean ± S.E.M. (n = 6 animals per group)

significant overall differences between groups ($F(3,36) = 20.22$, $p < 0.0001$) (Fig. 3b). Additionally, Tukey's post hoc analysis revealed significant differences between control and Sco groups ($p < 0.0001$), Sco and Sco + FEO1% groups ($p < 0.001$) and Sco and Sco + FEO3% groups ($p < 0.0001$) for SOD specific activity (Fig. 3b).

For the GPX specific activity estimated in the rat hippocampal homogenates, one-way ANOVA revealed a significant overall differences between groups ($F(3,36) = 6.64$, $p < 0.01$) (Fig. 3c). Additionally, Tukey's post hoc analysis revealed significant differences between control and Sco groups ($p < 0.01$), Sco and Sco + FEO1% groups ($p < 0.01$) and Sco and Sco + FEO3% groups ($p < 0.01$) for GPX specific activity (Fig. 3c).

For the CAT specific activity estimated in the rat hippocampal homogenates, one-way ANOVA revealed a significant overall differences between groups ($F(3,36) = 11.64$, $p < 0.001$) (Fig. 3d). Additionally, Tukey's post hoc analysis revealed significant differences between control and Sco groups ($p < 0.001$), control and Sco + FEO1% groups ($p < 0.01$), Sco and Sco + FEO1% groups ($p < 0.01$) and Sco and Sco + FEO3% groups ($p < 0.01$) for CAT specific activity (Fig. 3d).

Effect of the *F. angulata* Essential Oil on Total Content of Reduced GSH, Protein Carbonyl and MDA Levels

For the total content of reduced GSH estimated in the rat hippocampal homogenates, one-way ANOVA revealed a significant overall differences between groups ($F(3,36) = 57.92$, $p < 0.0001$) (Fig. 3e). Additionally, Tukey's post hoc analysis revealed significant differences between control and Sco groups ($p < 0.0001$), control and Sco + FEO1% groups ($p < 0.01$), Sco and Sco + FEO1% groups ($p < 0.0001$) and Sco and Sco + FEO3% groups ($p < 0.0001$) for the total content of reduced GSH (Fig. 3e).

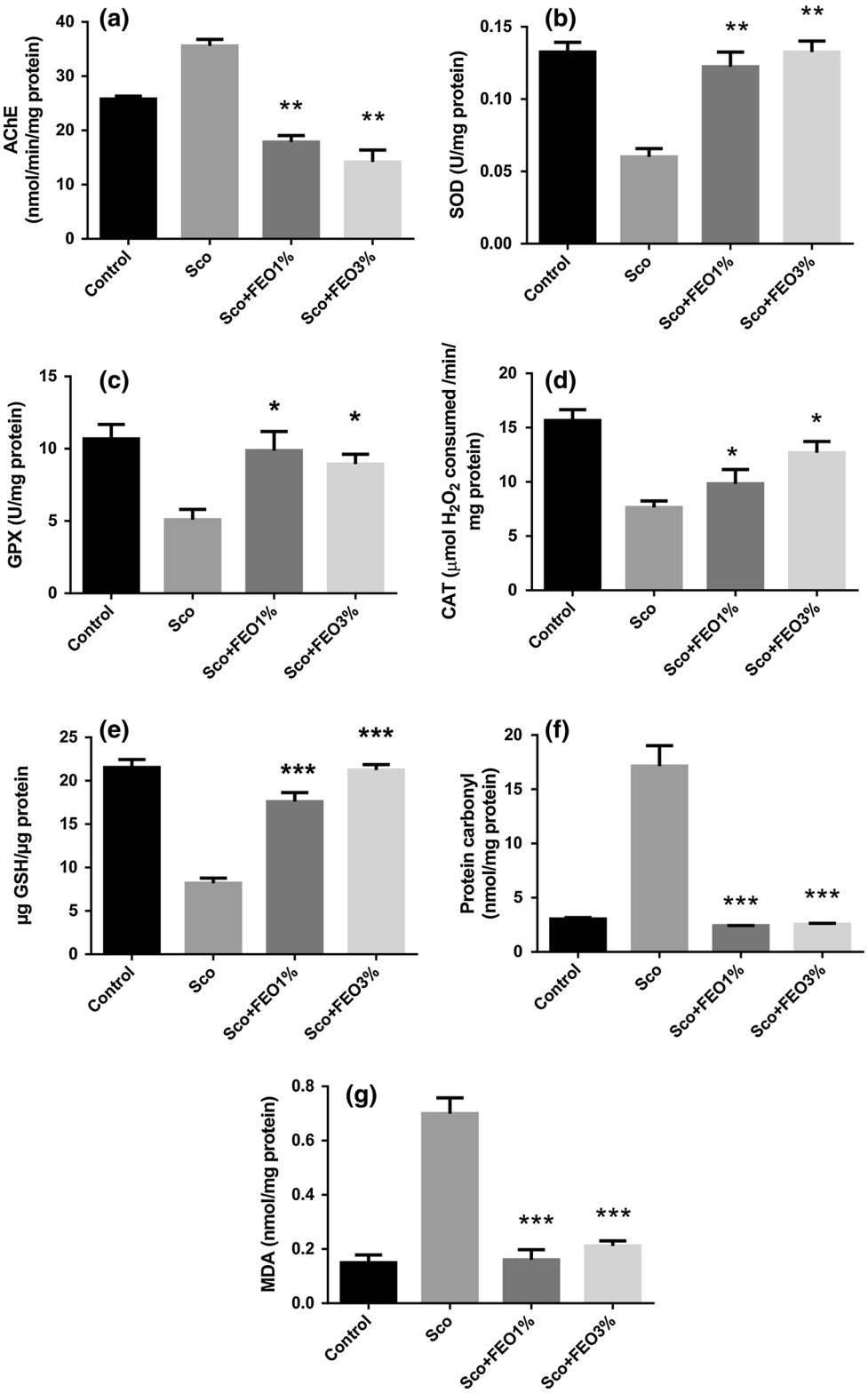
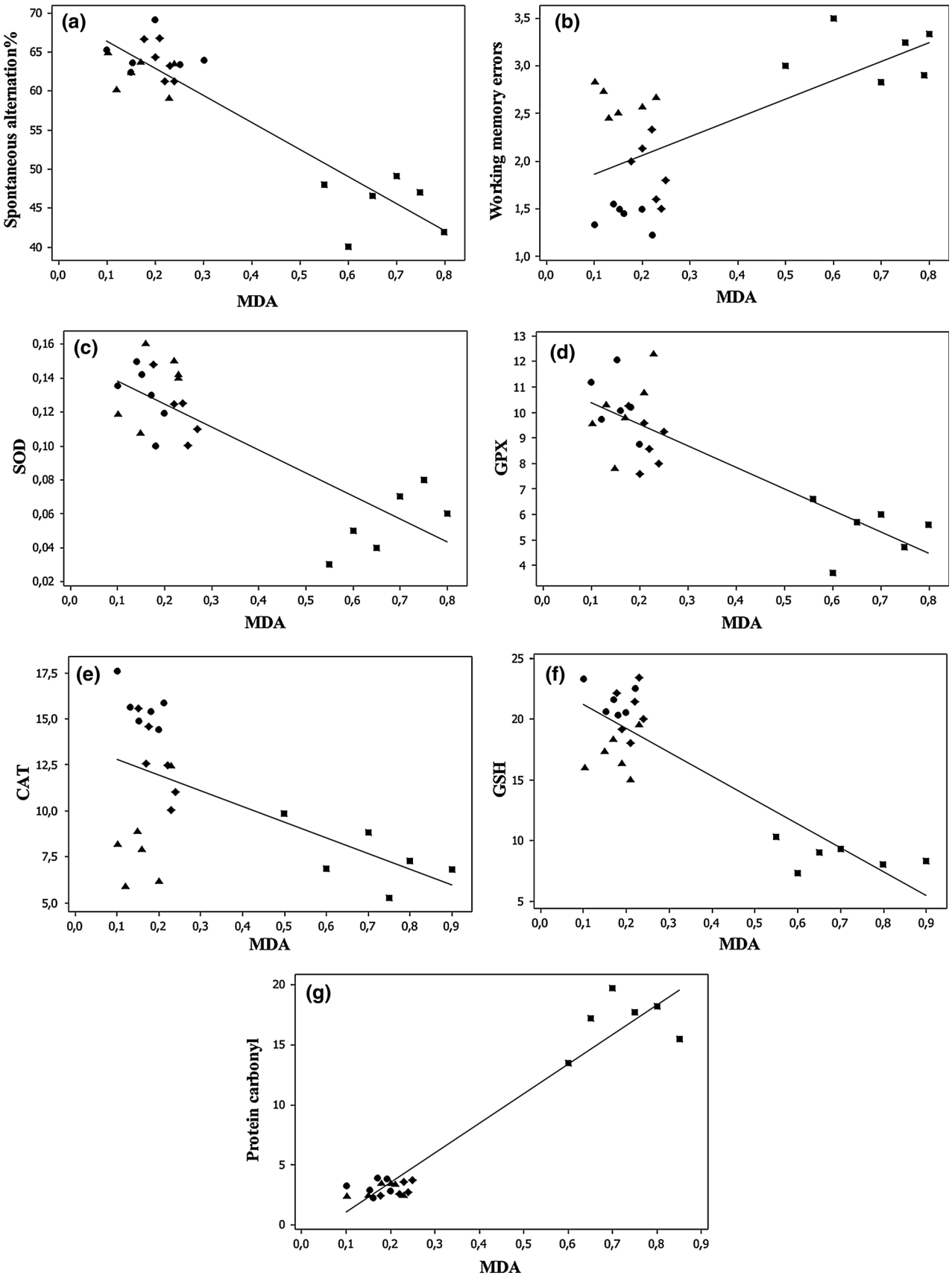


Fig. 3 Effects of inhaled *Ferulago angulata* essential oil (FEO1% and FEO3%) on AChE (**a**), SOD (**b**), GPX (**c**) and CAT (**d**) specific activities, on reduced GSH (**e**), protein carbonyl (**f**) and MDA (**g**) levels in the scopolamine (Sco)-treated rats. Values are mean ± S.E.M. (n = 6 animals per group), $*p < 0.01$, $**p < 0.001$, $***p < 0.0001$ versus scopolamine alone-treated group

For the protein carbonyl level estimated in the rat hippocampal homogenates, one-way ANOVA revealed a significant overall differences between groups ($F(3,36) = 57.91$, $p < 0.0001$) (Fig. 3f). Additionally, Tukey's post hoc analysis revealed significant differences between control and Sco groups ($p < 0.0001$), Sco and Sco + FEO1% groups ($p < 0.0001$) and Sco and Sco + FEO3% ($p < 0.0001$) for protein carbonyl level (Fig. 3f).


(a)
Spontaneous alternation%
MDA
(b)
Working memory errors
MDA
(c)
SOD
MDA
(d)
GPX
MDA
(e)
CAT
MDA
(f)
GSH
MDA
(g)
Protein carbonyl
MDA

◀**Fig. 4** Pearson's correlation between spontaneous alternation % versus MDA (**a**), working memory errors versus MDA (**b**), SOD versus MDA (**c**), GPX versus MDA (**d**), CAT versus MDA (**e**), GSH versus MDA (**f**) and protein carbonyl versus MDA (**g**) in control group *filled circle*, scopolamine (Sco) alone-treated group *filled square*, Sco + FEO1% group *filled diamond* and Sco + FEO3% group *filled triangle*

For the MDA level estimated in the rat hippocampal homogenates, one-way ANOVA revealed a significant overall differences between groups ($F(3,36) = 47.57$, $p < 0.0001$) (Fig. 3g). Additionally, Tukey's post hoc analysis revealed significant differences between control and Sco groups ($p < 0.0001$), Sco and Sco + FEO1% groups ($p < 0.0001$) and Sco and Sco + FEO3% groups ($p < 0.0001$) for MDA level (Fig. 3g).

These results support the hypothesis that the *F. angulata* essential oil may have induced a decrease in neuronal oxidative stress.

More importantly, when linear regression was determined, significant correlations between the spontaneous alternation percentage versus MDA ($n = 24$, $r = -0.923$, $p < 0.0001$) (Fig. 4a) and working memory errors versus MDA ($n = 24$, $r = 0.692$, $p < 0.01$) (Fig. 4b) were evidenced. Additionally, a significant correlation was evidenced by determination of the linear regression between SOD versus MDA ($n = 24$, $r = -0.884$, $p < 0.0001$) (Fig. 4c), GPX versus MDA ($n = 24$, $r = -0.777$, $p < 0.001$) (Fig. 4d), CAT versus MDA ($n = 24$, $r = -0.642$, $p < 0.01$) (Fig. 4e), GSH versus MDA ($n = 24$, $r = -0.892$, $p < 0.0001$) (Fig. 4f) and protein carbonyl versus MDA ($n = 24$, $r = 0.970$, $p < 0.0001$) (Fig. 4g).

These data suggest that improving of spatial memory formation within the Y-maze and the radial arm-maze tasks and the increase of the antioxidant defence and the decrease of AChE activity along with the decrease of lipid peroxidation and protein oxidation could related with the involvement of the *F. angulata* essential oil in neuroprotection against scopolamine-induced neuronal oxidative stress generation.

Discussion

The present study aimed to examine the spatial memory formation following inhalation of the *F. angulata* essential oil (1 and 3 %, for 21 continuous days) in rats subjected to i.p. injection of scopolamine. Consequently, i.p. injection of scopolamine causes memory impairment, in accordance with previous investigations [31–33].

In the present study we used two well-characterized hippocampus-dependent spatial memory tasks: Y-maze and radial arm-maze. These behavioral tasks can test hippocampus-dependent short-term, long-term and spatial memory processing, which are particularly affected by AD [34]. Our results clearly demonstrated that inhalation of the *F. angulata* essential oil sustains spatial memory formation in a rat model of scopolamine-induced learning and memory impairment.

The GC–MS/GS-FID analysis indicated monoterpenes, including α-pinene (24.10 %), β-pinene (22.70 %), α-phellandrene (12.10 %) and β-phellandrene (20.50 %) as the main components of our *F. angulata* essential oil suggesting that these constituents could be responsible for the observed memory-enhancing effects in scopolamine-treated rats. Among them, α-pinene was identified in high amount and it has been reported to present an AChE inhibitory effect [35]. Both major components of the essential oil, such as α-pinene and β-pinene, were tested for their potential neuroprotective effects [36, 37].

The high dose of *F. angulata* essential oil in scopolamine-treated rats significantly improved spatial working memory in the Y-maze task (Fig. 1a), as evidenced by the increase of spontaneous alternation percentage as compared to scopolamine alone-treated rats. This result suggests that the high dose of *F. angulata* essential oil (3 %) used in this study displays an improved effect on acquisition of the short-term memory of scopolamine-treated rats within the Y-maze task. However, no differences were observed between both doses of *F. angulata* essential oil on spatial working memory within the Y-maze task. The improvement of spatial working memory within Y-maze task cannot be attributed to locomotor activity, because significant changes in the number of entries of the groups treated with the *F. angulata* essential oil as compared with scopolamine alone-treated rats were observed (Fig. 1b).

Scopolamine-treated rats exposed to high dose of *F. angulata* essential oil (3 %) exhibited an improvement of working memory in the radial arm-maze task as compared with scopolamine alone-treated rats (Fig. 2a). On the other hand, both doses of inhaled *F. angulata* essential oil (1 and 3 %) non-significantly improved long-term memory of scopolamine-treated rats, explored by reference memory within the radial arm-maze task (Fig. 2b). These findings suggest that inhalation of the *F. angulata* essential oil plays an important role in spatial memory formation, especially on working memory. However, non-significant differences were observed between both doses of the *F. angulata* essential oil on working memory and reference memory in the radial arm-maze task.

Previous studies have described that scopolamine-induced memory impairment is an appropriate model for the study of the cholinergic dysfunction in both neurotransmitters and their receptors [38, 39]. Acetylcholine (ACh) levels in the central cholinergic synapses need to be maintained to normalize memory function. Moreover, the

disruption of ACh by the overexpression of AChE can disrupt cholinergic activity in the synapse [40]. Our results indicated that the hippocampal AChE activity was significantly increased by scopolamine (Fig. 3a). Inhalation of the *F. angulata* essential oil significantly decreased the hippocampal AChE activity of scopolamine treated-rats in a dose-dependent manner. As AChE inhibitors have shown potential in rodent models of amnesia [41], the attenuation of the hippocampal AChE activity suggested that the *F. angulata* essential oil may confer anti-amnesic effects on scopolamine-induced memory impairment in rats.

The present study also evaluated whether memory impairment induced by scopolamine is related with altered oxidative stress indices. Scopolamine alone-treated rats had reduced SOD (Fig. 3b), GPX (Fig. 3c) and CAT (Fig. 3d) specific activities, along with decreased content of reduced GSH (Fig. 3e) and elevated protein carbonyl (Fig. 3f) and MDA (Fig. 3g) levels within the hippocampal homogenates. The decreases of the SOD and CAT specific activities appeared to parallel increases in the protein carbonyl and the MDA levels in the hippocampal homogenates suggesting that these events are needed to scavenge superoxide radicals induced by scopolamine. Protein oxidation is an important factor in aging and age-related neurodegenerative disorders and is indexed by the presence of protein carbonyls [42]. MDA is the most abundant individual aldehyde resulting from lipid peroxidation and can be considered a marker of lipid peroxidation [19]. Furthermore, many studies have reported the strong positive correlation that memory impairments in the scopolamine-induced amnesic rats show similar patterns of oxidative damage in the patients with amnesic MCI [43].

Treatment of scopolamine-induces amnesic rats with the *F. angulata* essential oil significantly decreased the protein carbonyl and the MDA level and restored the specific activities of SOD, GPX and CAT, and also the total content of reduced GSH in the hippocampal homogenates. Our results indicated that the *F. angulata* essential oil possesses potent antioxidant activity by scavenging reactive oxygen species (ROS) and exerting a protective effect against oxidative stress induced by scopolamine.

Moreover, we found a significant correlation between spontaneous alternation percentage versus MDA, working memory errors versus MDA, SOD versus MDA, GPX versus MDA, CAT versus MDA, GSH versus MDA, protein carbonyl versus MDA when linear regression was determined. These results could suggest that the increase of behavioral parameters in the Y-maze and the radial arm-maze tasks and the antioxidant defence along with the decrease of lipid peroxidation and the protein oxidation could be correlated with the involvement of the *F. angulata* essential oil in neuroprotection against scopolamine-induced oxidative stress generation in the rat hippocampus.

In conclusion, the present study indicated that the *F. angulata* essential oil could effectively enhance memory processes, decreases the activity of the cholinergic enzyme such as AChE, restore antioxidant brain status and may confer neuroprotection due to alleviation of oxidative damage induced by scopolamine. These results suggest that the *F. angulata* essential oil may possibly be used as an effective agent to prevent cholinergic dysfunction, such as AD.

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Compliance with Ethical Standards

Conflict of interests The authors have declared that no competing interests exist.

Ethical standard This study was approved by the Committee on the Ethics of Animal Experiments of the Alexandru Ioan Cuza University of Iasi (Permit Number: 2192) and also, efforts were made to minimize animal suffering and to reduce the number of animals used.

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